Electrophoresis Techniques

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Theory of electrophoresis

- **Introduction:**

Electrophoresis is a physical method of analysis which involves separation of the compounds that are capable of acquiring electric charge in conducting electrodes.

- **Definition:**

Electrophoresis may be defined as the migration of the charged particle through a solution under the influence of an external electrical field.

Ions that are suspended between two electrodes tends to travel towards the electrodes that bears opposite charges.

Father of Electrophoresis

Arne Tiselius

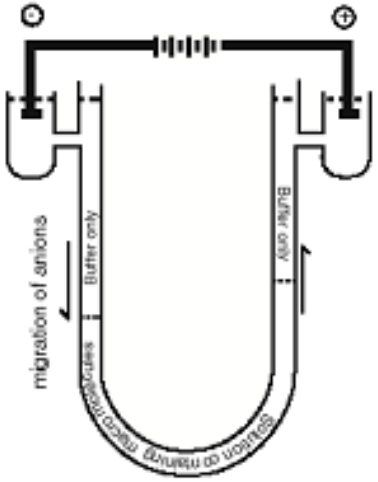
(Sweden, 1902-1971)

The Nobel Prize in Chemistry 1948

"for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins"

This type of cell is essentially a bent glass tube with electrolyte reservoirs containing the cathode and anode, and a buffer containing the macromolecules that need electrophoresed.

He tested horse serum in the apparatus and found 4 distinct bands consisting of albumin and 3 globulin components, which he named "α," "β," and "γ."



Theory of electrophoresis

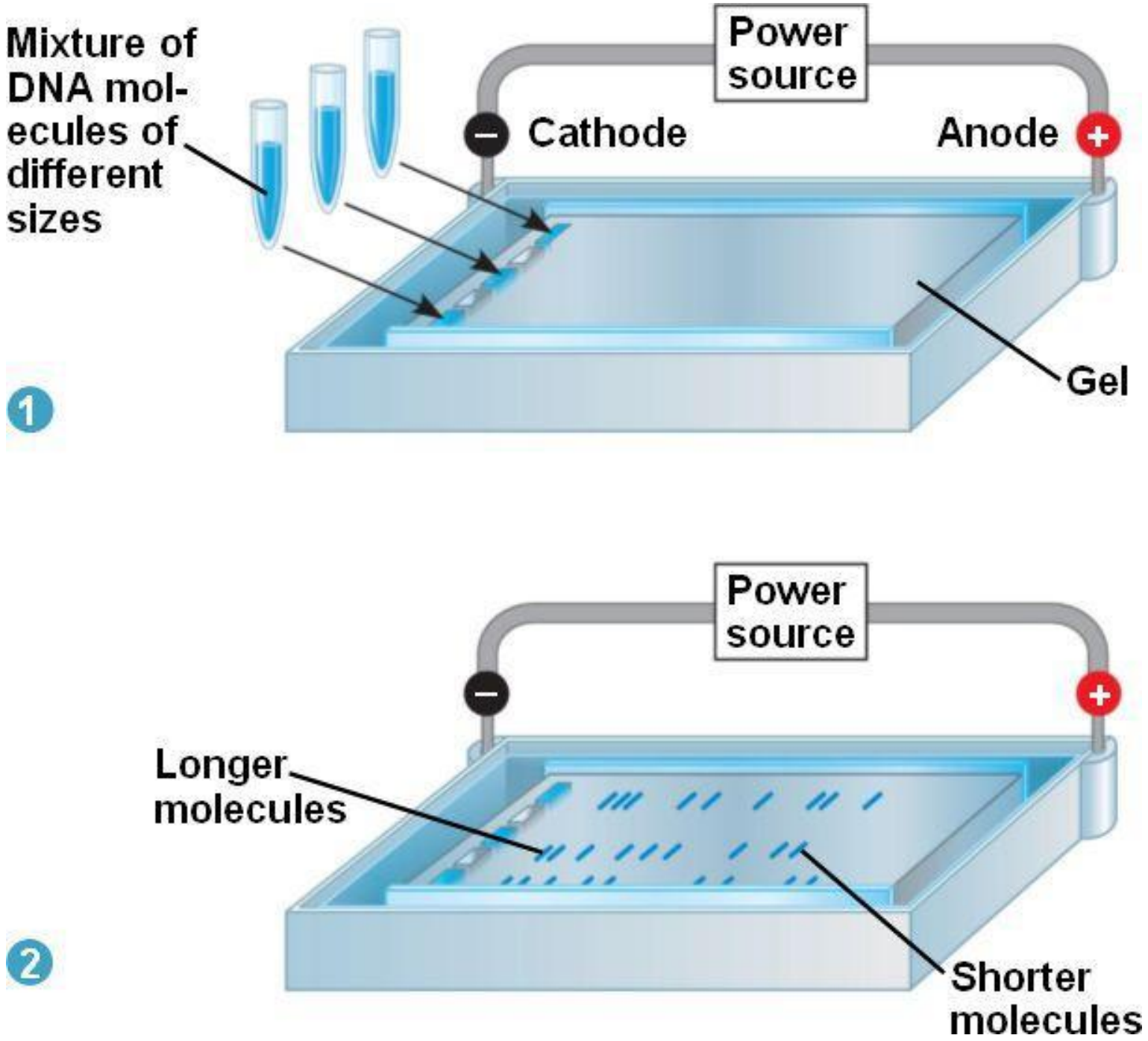
The rate of migration of an ion in electrical field depend on factors:

1. Net charge of molecule
2. Size and shape of particle
3. Strength of electrical field
4. Properties of supporting medium
5. Temperature of operation

Theory of electrophoresis

Mobility

- Under the electrical field, the mobility of the particle is determined by two factors:
  - 1- Its charge
  - 2- Frictional coefficient
- Size and shape of the particle decide the velocity with which the particle will migrate under the given electrical field and the medium.


Mixture of DNA molecules of different sizes
Power source
Cathode
Anode
Gel
1
Power source
Longer molecules
Shorter molecules
2

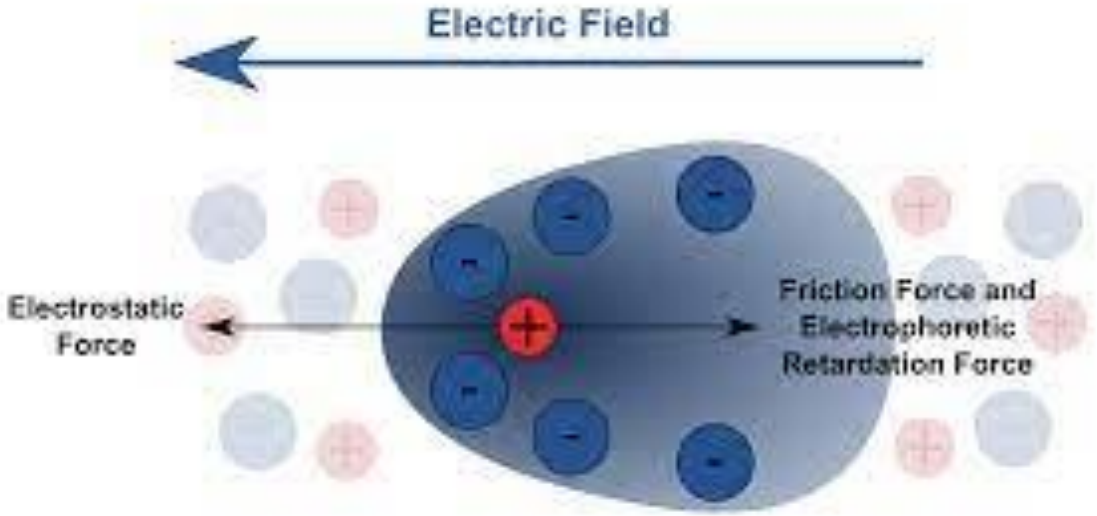
Theory of electrophoresis

Strength of electrical field

- It determined by the force exerted on the particle, and the charge the particle carrying.

F=QV

when force is exerted on the particle it start moving, however the movement is restricted by the experience of the frictional force because of the viscosity.



Theory of electrophoresis

Effect of pH on Mobility

- As the molecule exist as amphoteric , they will carry the charges based on the solvent pH.
- Their overall net charge is NEUTRAL when it is at zwitter ion state. And hence the mobility is retarded to zero.
- Mobility is directly proportional to the magnitude of the charge, which is functional of the pH of solvent.
- The pH is maintained by the use of Buffers of different pH.

Theory of electrophoresis

Electrophoretic velocity depends on:

Inherent Factors

- Magnitude of its charge
- Charge density
- Molecular weight
- Tertiary or quaternary structure (i.e., its shape).

External environment

- Solution pH
- Electric field
- Solution viscosity
- temperature

Electrophoresis

- A separation technique
- Simple, rapid and highly sensitive
- used in clinical laboratories to separate charged molecules from each other in presence of electric field

✓ Serum Protein Electrophoresis

✓ Lipoprotein Analysis

✓ Diagnosis of Haemoglobinopathies and Haemoglobin A1c

Clinical applications of Electrophoresis

- ✓ Determination of Serum Protein Phenotypes and Micro heterogeneities e.g. α1- antitrypsin deficiency, MM
- ✓ Genotyping of Proteins eg. ApoE analysis for Alzheimer's disease (polymorphic protein)
- ✓ Small Molecules (Drugs, Steroids) Monitoring
- ✓ Cerebrospinal Fluid Analysis
- ✓ Urine Analysis (determination of glomerulonephritis)
- ✓ Nucleic acids: DNA, RNA

Types of electrophoresis

Zone electrophoresis

a) Paper electrophoresis
b) Gel electrophoresis
c) Thin layer electrophoresis
d) Cellulose acetate electrophoresis

Moving Boundary Electrophoresis

a) Capillary Electrophoresis
b) Isotachophoresis
c) Isoelectric Focusing
d) Immuno Electrophoresis

Zone electrophoresis

- It involves the migration of the charged particle on the supporting media (Paper, cellulose acetate membrane, starch gel, poly acrylamide).
- The separated components are distributed into discrete zone on the support media.
- Supporting media is saturated with buffer solution, small volume of the sample is applied as narrow band.

Zone electrophoresis

Advantages

- Useful in biochemical investigations.
- Small quantity of sample can be analyzed.
- Low cost and easy maintenance.

Disadvantages

- Unsuitable for accurate mobility and isoelectric point determination.
- Due to the presence of supporting medium, technical complications such as capillary flow, electro osmosis, adsorption and molecular sieving are introduced.

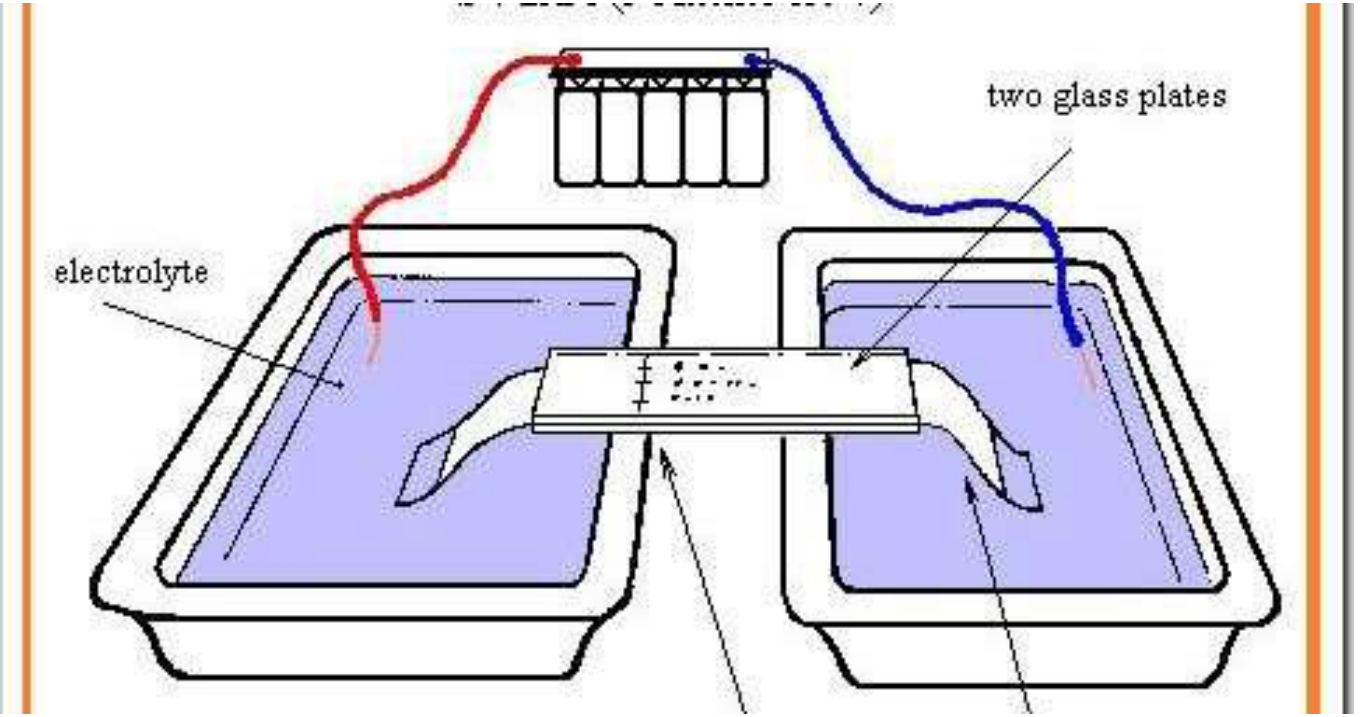
General method of operation

- Saturation of the medium with the buffer.
- Sample application.
- Electrophoretic separation.
- Removal of the supporting media.

Instrumentation

- Electrophoresis chamber.
- Electrodes.
- Diffusion barriers.
- Supporting / stabilizing media. (inert to sample and to any developing reagents)

Paper electrophoresis



1. Horizontal paper Electrophoresis
2. Vertical paper Electrophoresis

Paper electrophoresis

- Filter paper such as Whatmann no1 and no3 in strip of 3 mm or 5 cm wide have been used to good effect.
- Separation takes place in 12 to 14 hrs.

Advantages

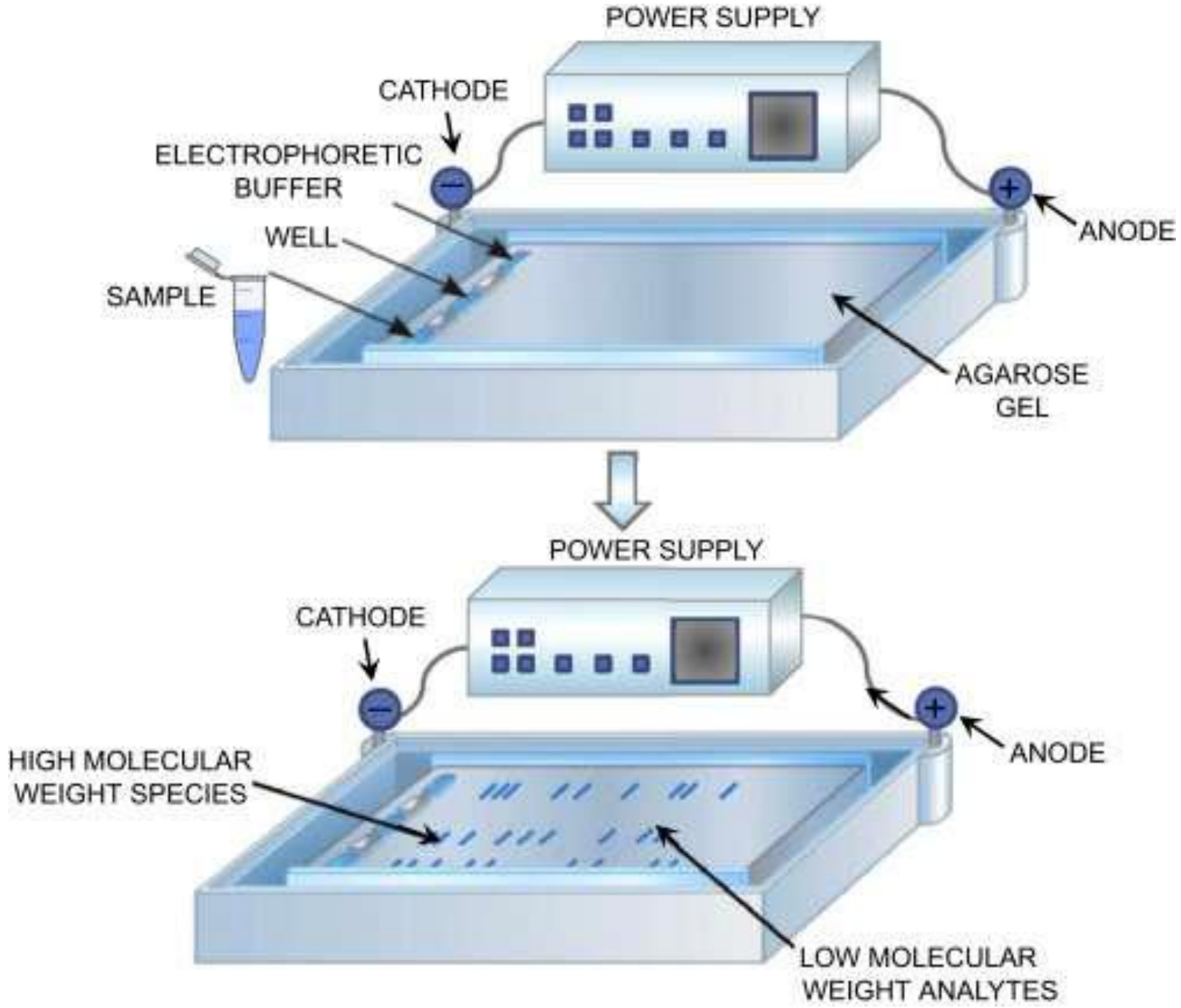
- It is economical
- Easy to use

Disadvantages

- Certain compounds such as proteins, hydrophilic molecules cannot be resolved due to the adsorptive and ionogenic properties of paper which results in tailing and distortion of component bands.
- Electro osmosis

Gel electrophoresis

- Separation is brought about through molecular sieving technique, based on the molecular size of the substances.
- Gel material acts as a “molecular sieve”
- Gel is a colloid in a solid form (99% is water).
- It is important that the support media is electrically neutral.
- Different types of gels which can be used are; Agar and Agarose gel, Starch, Sephadex, Polyacrylamide gels.
- A porous gel acts as a sieve by retarding or, in some cases, by completely obstructing the movement of macromolecules while allowing smaller molecules to migrate freely.


POWER SUPPLY
CATHODE
ELECTROPHORETIC BUFFER
WELL
SAMPLE
ANODE
AGAROSE GEL
POWER SUPPLY
CATHODE
ANODE
HIGH MOLECULAR WEIGHT SPECIES
LOW MOLECULAR WEIGHT ANALYTES

Agar and agarose gel

- Agar is a mixture of polysaccharides extracted from sea weeds.
- Agarose is a highly purified uncharged polysaccharide derived from agar.
- Agarose is chemically basic disaccharide repeating units of 3,6-anhydro-L-galactose.
- Agarose dissolves when added to boiling liquid. It remains in a liquid state until the temperature is lowered to about 40 °C at which point it gels.
- The pore size may be predetermined by adjusting the concentration of agarose in the gel.
- Agarose gels are fragile. They are actually hydrocolloids, and they are held together by the formation of weak hydrogen and hydrophobic bonds.
- The pores of an agarose gel are large, agarose is used to separate macromolecules such as nucleic acids, large proteins and protein complexes

Agar and agarose gel

Advantages

- Easy to prepare and small concentration of agar is required.
- Resolution is superior to that of filter paper.
- Large quantities of proteins can be separated and recovered.
- Adsorption of negatively charged protein molecule is negligible.
- It adsorbs proteins relatively less when compared to other medium.
- Sharp zones are obtained due to less adsorption.
- Recovery of protein is good, good method for preparative purpose

Disadvantages

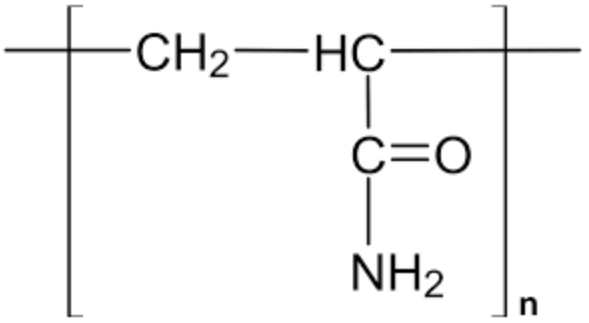
- Electro osmosis is high.
- Resolution is less compared to polyacrylamide gels.
- Different sources and batches of agar tend to give different results and purification is often necessary.

Application

- Widely used in immuno electrophoresis

Polyacrylamide gel electrophoresis (PAGE)

- It is prepared by polymerizing acryl amide monomers in the presence of methylene-bis-acrylamide to cross link the monomers.
- Structure of acrylamide (CH2=CH-CO-NH2)
- Polyacrylamide gel structure held together by covalent cross-links.
- Polyacrylamide gels are tougher than agarose gels.
- It is thermostable, transparent, strong and relatively chemically inert.
- Gels are uncharged and are prepared in a variety of pore sizes.
- Proteins are separated on the basis of charge to mass ratio and molecular size, a phenomenon called Molecular sieving.



Advantage

- Gels are stable over wide range of pH and temperature.
- Gels of different pore size can be formed.
- Simple and separation speed is good comparatively.

Types of PAGE

NATIVE-PAGE

- Native gels are run in non-denaturing conditions, so that the analyte's natural structure is maintained.
- Separation is based upon charge, size, and shape of macromolecules.
- Useful for separation or purification of mixture of proteins.
- This was the original mode of electrophoresis

DENATURED-PAGE OR SDS-PAGE

- Separation is based upon the molecular weight of proteins.
- The common method for determining MW of proteins.
- Very useful for checking purity of protein samples

PAGE-procedure

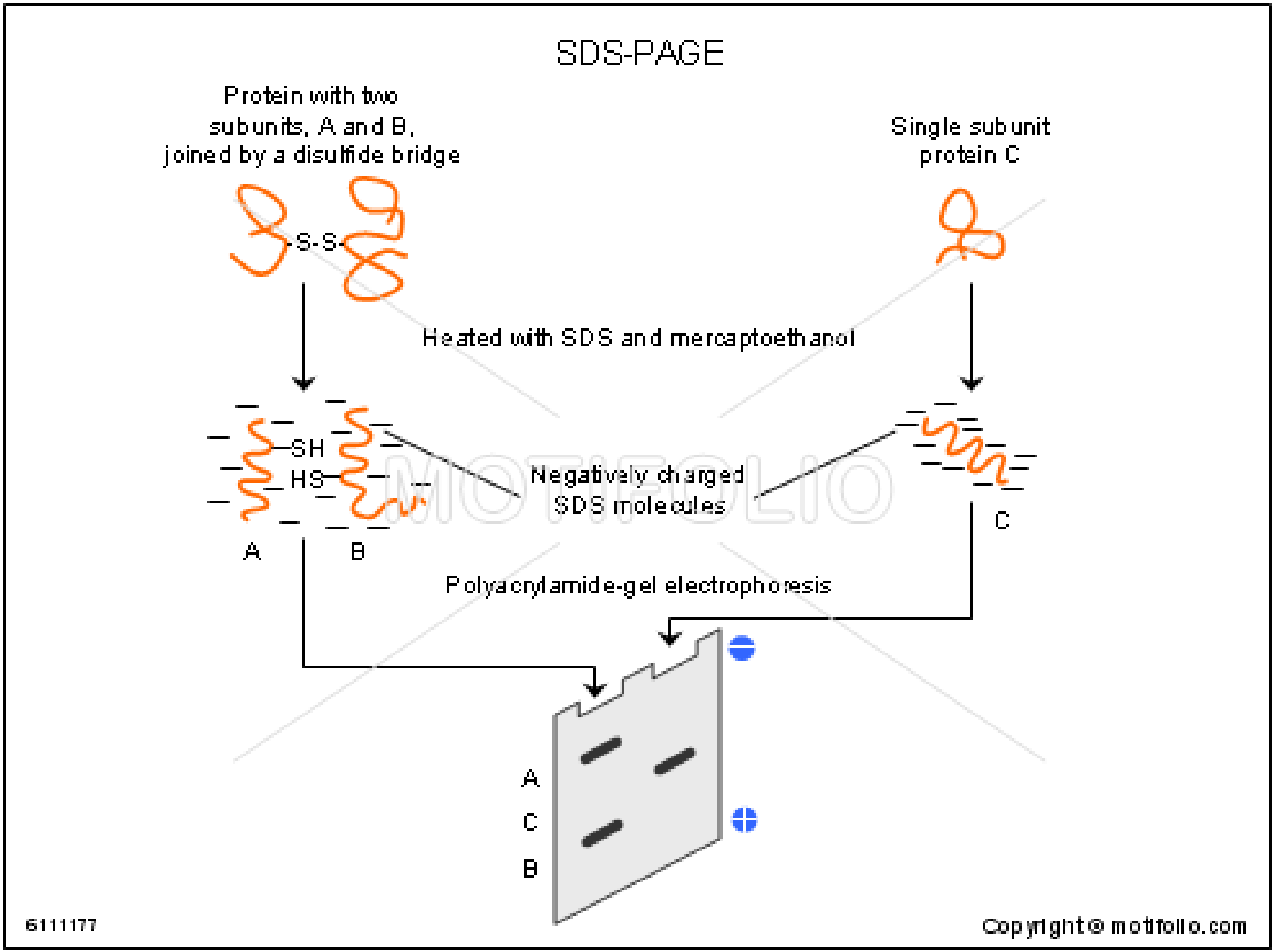
- The gel of different pore sizes is cast into a column inside a vertical tube, often with large pore gel at the top and small pore gel at the bottom.
- Microgram quantity of the sample is placed over the top of the gel column and covered by a buffer solution having such a pH so as to change sample components into anions.
- The foot of the gel column is made to dip in the same buffer in the bottom reservoir.
- Cathode and anode are kept above and below the column to impose an electric field through the column.
- Macromolecular anions move towards the anode down the gel column.
- There is no external solvent space, all the migratory particles have to pass through the gel pores.
- Rate of migration depends on the charge to mass ratio.
- Different sample components get separated into discrete migratory bands along the gel column on the basis of electrophoretic mobility and gel filtration effect.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

- SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility.
- When a detergent SDS added to PAGE the combined procedure is termed as SDS PAGE.
- SDS coats protein molecules giving all proteins a constant charge-mass ratio.
- Due to masking of charges of proteins by the large negative charge on SDS binding with them, the proteins migrate along the gel in order of increasing sizes or molecular weights.
- SDS is an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures by wrapping around the polypeptide backbone. In so doing, SDS confers a net negative charge to the polypeptide in proportion to its length.
- Molecules in solution with SDS have a net negative charge within a wide pH range.
- A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass.
- The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode in an electric field.

Sodium dodecyl sulfate (SDS-PAGE)

- Native protein is unfolded by heating in the presence of mercaptoethanol and SDS.
- SDS binds to the protein so that it stays in solution and denatures.
- Large polypeptides bind more SDS than small polypeptides, so proteins end up with negative charge in relation to their size.
- When treated with SDS and a reducing agent, the polypeptides become rods of negative charges with equal “charge densities" or charge per unit length.
- Thus, we can separate the proteins based on their mass


SDS-PAGE
Protein with two subunits, A and B, joined by a disulfide bridge
Single subunit protein C
S-S
Heated with SDS and mercaptoethanol
SH
HS
Negatively charged SDS molecules
A
B
C
Polyacrylamide-gel electrophoresis
A
C
B
6111177
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Starch

- A suspension of granular starch should be boiled in a buffer to give a clear colloidal suspension.
- The suspension on cooling sets as a semisolid gel due to intertwining of the branched chains of amylopectin.
- In order to avoid swelling and shrinking petroleum jelly is used.

Advantages

- High resolving power and sharp zones are obtained.
- The components resolved can be recovered in reasonable yield especially proteins.
- Can be used for analytical as well as preparative electrophoresis.

Disadvantages

- Electro osmotic effect.
- Variation in pore size from batch to batch

Thin layer electrophoresis

Studies can be carried out in thin layer of silica, alumina.

Advantages

- Less time consuming and good resolution.

Application

- Widely used in combined electrophoretic-chromatography studies in two dimensional study of proteins and nucleic acid hydrolysates

Cellular acetate electrophoresis

- It contains 2-3 acetyl groups per glucose unit and its adsorption capacity is less than that of paper.
- It gives sharper bands.
- Provides a good background for staining glycoproteins.

Advantages

- No tailing of proteins or hydrophilic materials.
- Available in wide range of particle size and layer thickness.
- Give sharp bands and offer good resolution.
- High voltage can be applied which will enhance the resolution.

Disadvantages

- Expensive.
- Presence of sulphonic and carboxylic residue causes induced electro osmosis during electrophoresis.

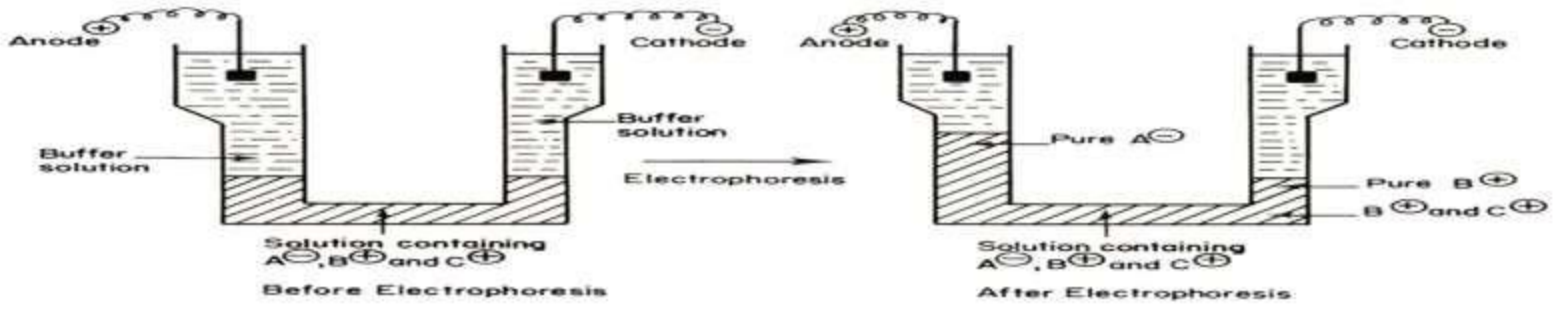
Application

- Widely used in analysis of clinical and biological protein samples (albumin and globulins).
- Alternative to paper electrophoresis.

Moving boundary electrophoresis

PRINCIPLE:

The moving boundary method allows the charged species to migrate in a free moving solution without the supporting medium



Moving boundary electrophoresis.

Moving boundary electrophoresis

Advantages

- Biologically active fractions can be recovered without the use of denaturing agents.
- A reference method for measuring electrophoretic motilities.

Disadvantages

- Costly
- Elaborate optical system are required.

Application

- To study homogenecity of a macromolecular system.
- Analysis of complex biological mixtures

Capillary electrophoresis

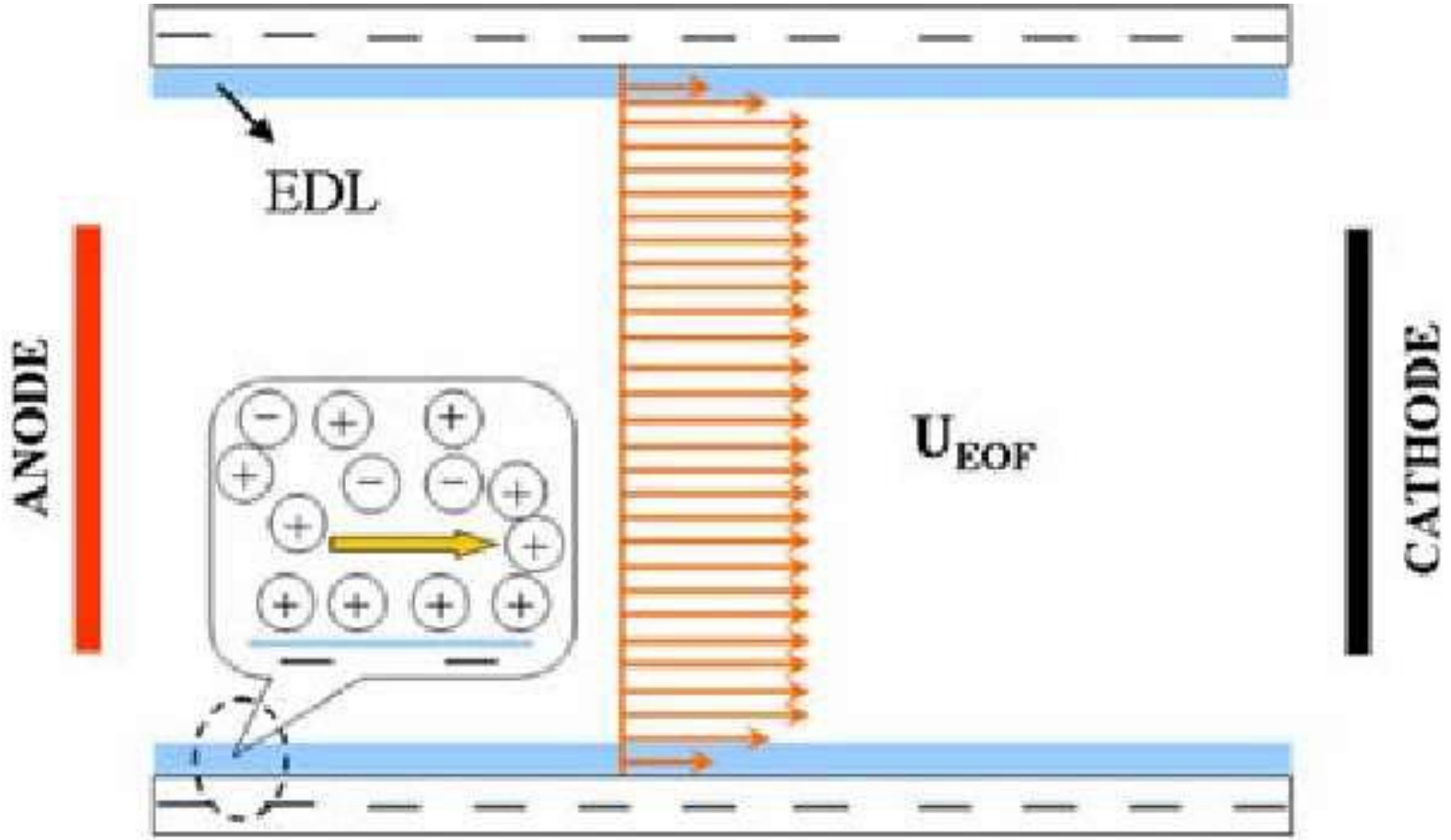
- The principle behind electrophoresis is that charged molecules will migrate toward the opposite pole and separate from each other based on physical characteristics.
- Capillary electrophoresis has grown to become a collection of a range of separation techniques which involve the application of high voltages across buffer filled capillaries to achieve separations .
- Capillary electrophoresis, then, is the technique of performing electrophoresis in buffer filled, narrow-bore capillaries, normally from 25 to 100 mm in internal diameter (ID).
- A high voltage (typically 10-30 kV) is applied.
- Capillaries are typically of 50 µm inner diameter and 0.5 to 1 m in length.
- Due to electro osmotic flow, all sample components migrate towards the negative electrode.
- The capillary can also be filled with a gel, which eliminates the electro osmotic flow. Separation is accomplished as in conventional gel electrophoresis but the capillary allows higher resolution, greater sensitivity, and on-line detection.
- The capillary is filled with electrolyte solution which conducts current through the inside of the capillary. The ends of the capillary are dipped into reservoirs filled with the electrolyte.
- Electrodes (platinum) are inserted into the electrolyte reservoirs to complete the electrical circuit.

Electroosmotic flow

The surface of the silicate glass capillary contains negatively-charged functional groups that attract positively-charged counter ions. The positively-charged ions migrate towards the negative electrode and carry solvent molecules in the same direction. This overall solvent movement is called electro osmotic flow. During a separation, uncharged molecules move at the same velocity as the electro osmotic flow (with very little separation). Positively-charged ions move faster and negatively-charged ions move slower.

A small volume of sample is moved into one end of the capillary. The capillary passes through a detector, (usually a UV absorbance detector), at the opposite end of the capillary.

- Application of a voltage causes movement of sample ions towards their appropriate electrode usually passing through the detector.
- A plot of detector response with time is generated which is termed an electropherogram


EDL
ANODE
CATHODE
U_{EOF}

Isotachophoresis

The technique of isotachophoresis depends on the development of potential gradient.

Principle:

- Based on principle of moving boundary electrophoresis. A leading electrolyte(e.g. chloride) with a higher mobility than the analytes, and a trailing electrolyte(e.g. glycinate) with a lower mobility are used.
- Solution in which the separation takes place is normally an aqueous medium, which contains sucrose to provide a higher density to the solution.
- Where the separation by Isoelectric focusing depends on the existence of a pH gradient in the system. The technique of Isotachophoresis depends on the development of a potential gradient.
- Separation of the ionic components of the sample is achieved through stacking them into discrete zones in order of their mobilities , producing very high resolution.

Isotachophoresis

- The analyte are positioned between the electrolytes and, when the voltage is applied, they migrate in order of decreasing mobility.
- This establishes the potential gradient; from that point on, all the analyte move at the same speed.
- Individual zones border one another but represent completely separated components without overlap.
- In isotachophoresis no background electrolyte(buffer) is mixed with the sample, so current flow is carried only by charged sample ions.
- Once a faster moving component separates completely from a slower moving one, It creates a region of depleted charge between the two that increases the resistance and therefore local voltage in that region.
- This increased voltage causes the slower component to migrate faster and close the gap, thereby concentrating it and increasing the conductivity of its zone until it matches that of the faster ion.
- Ultimately all ions migration at the rate of the faster ion in the zones that differ in thickness, depending on their original concentrations.

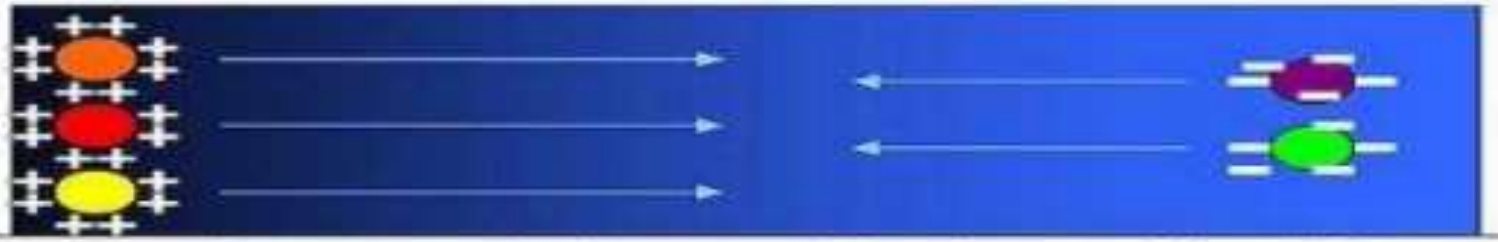
Application

- Isotachophoresis that been used for the separation of proteins as well as inorganic substances.

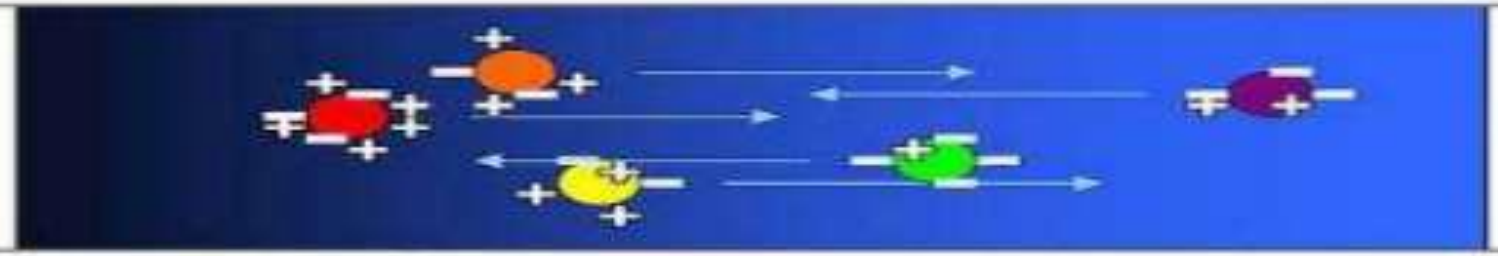
Isoelectric focusing

- All proteins have an isoelectric point pH .
- When electrophoresis is run in a solution buffered at constant pH , proteins having a net charge will migrate towards the opposite electrode so long as the current flows.
- The use of pH gradient across the supporting medium causes each protein to migrate to an area of specific pH. The pH of the protein equals the pH of the gradient, thus resulting in sharp well defined protein bands.
- A procedure to determine the isoelectric point (PI) of proteins thus, a mixture of proteins can be electrophorised through a solution having a stable pH gradient from the anode to the cathode and each protein will migrate to the position in the pH gradient according to its isoelectric point. This is called isoelectric focusing.
- Protein migrate into the point where its net charge is zero – isoelectric pH.
- Protein is positively charged in solutions at pH below its PI and will migrate towards the cathode.
- Protein is negatively charged in solution at pH above its PI will migrate towards the anode.
- They will be in the Zwitter ion form with no net charge so the further movement will cease.
- Ampholytes (amphoteric electrolytes)- low molecular mass (600-900D) oligomers with aliphatic amino and carboxylic acid groups with a range of isoelectric points. Ampholytes help maintain the pH gradient in the presence of high voltage.
- Can also use gels with immobilized pH gradients - made of acrylamide derivatives that are covalently linked to ampholytes.

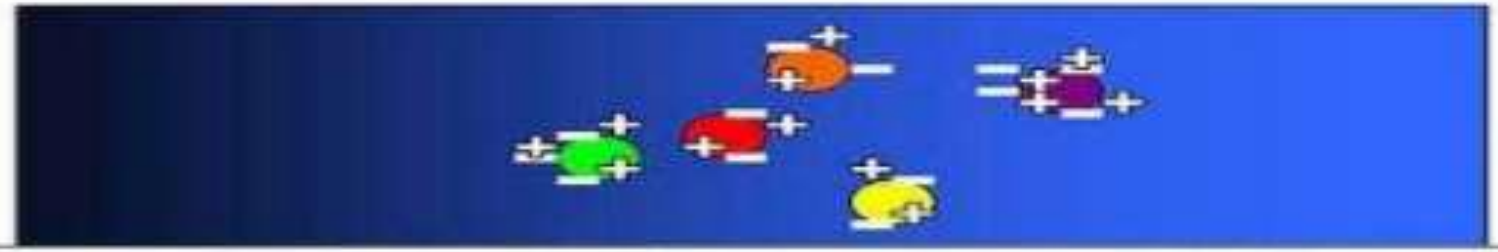
Stable pH gradient



At low pH, most proteins have a positive charge while at high pH, most proteins have a negative charge.



When an electric field is present, the cathode and anode ends pull the proteins to their isoelectric point where each individual protein possesses a neutral charge.



The proteins stopped migrating because they've reached their isoelectric point at a unique pH level.

Advantages

- As spreading of bands is minimized due to application of the applied field and the PH gradient , high resolution can be achieved.
- Proteins that differ by as little as 0.001 PH units can be separated.

Disadvantages

- Because carrier ampholytes are generally used in high concentration, a high voltage (up to 2000v) is necessary . As a result the electrophoretic matrix must be cooled which sometimes makes it difficult.

Applications

- For separating proteins and peptides.
- For research in enzymology , immunology, cytology and taxonomy.


THANK YOU